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PRINCIPAL INVESTIGATOR: Louis M. Weiner, M.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

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6. AUTHOR(S)
Louis M. Weiner, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

E-Mail: LM_Weiner@fccc.edu

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We hypothesized that the selective accumulation of systemically administered cytokines at tumor sites can alter tumor microenvironments to favor the induction of anti-tumor immune responses. We further hypothesized that this can be accomplished by pre-targeting tumors with antibody-streptavidin immunoconjugates and then administering biotinylated cytokines. The purpose of this research program was to identify antibody-pretargeted cytokine therapy strategies that lead to tumor-selective cytokine accumulation, the development of host inflammatory cell infiltrates in tumor, and the induction of tumor-specific immunity. The ultimate goal of this research was to identify candidate strategies for clinical development, alone or in combination with tumor vaccines. In the first year of this award we made significant progress toward achieving these goals. Interleukin-2 (IL-2) was biotinylated, and its biological properties were thoroughly characterized. We obtained a streptavidin-conjugated monoclonal antibody that recognized the Ep-CAM tumor antigen that is frequently overexpressed in breast cancer specimens. Animal experiments to characterize the biodistribution properties of the antibody – streptavidin conjugate and of the conjugate admixed with biotinylated IL-2 were performed in year 2. Because biodistribution results did not suggest that the previously used streptavidin-biotin system would yield therapeutic results we redirected efforts in the final year on developing new systems employing bispecific minibodies that contain anti-tumor binding domains and domains that bind to a metal chelate that can serve as a universal acceptor for metal-conjugated cytokines.

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INTRODUCTION

We hypothesized that the selective accumulation of systemically administered cytokines at tumor sites can alter tumor microenvironments to favor the induction of anti-tumor immune responses. We further hypothesized that this can be accomplished by pre-targeting tumors with antibody-streptavidin immunoconjugates and then administering biotinylated cytokines. The purpose of this research program was to identify antibody-pretargeted cytokine therapy strategies that lead to tumor-selective cytokine accumulation, the development of host inflammatory cell infiltrates in tumor, and the induction of tumor-specific immunity. The ultimate goal of this research was to identify candidate strategies for clinical development, alone or in combination with tumor vaccines. In the first year of this award we made significant progress toward achieving these goals. Interleukin-2 (IL-2) was biotinylated, and its biological properties were thoroughly characterized. We obtained a streptavidin-conjugated monoclonal antibody that recognized the Ep-CAM tumor antigen that is frequently overexpressed in breast cancer specimens. Animal experiments to characterize the biodistribution properties of the antibody – streptavidin conjugate and of the conjugate admixed with biotinylated IL-2 were performed in year 2. Because biodistribution results did not suggest that the previously used streptavidin-biotin system would yield therapeutic results we redirected efforts in the final year on developing new systems employing bispecific minibodies that contain anti-tumor binding domains and domains that bind to a metal chelate that can serve as a universal acceptor for metal-conjugated cytokines.

BODY

Technical Objectives

1. To determine the conditions required to selectively pretarget streptavidin to human tumor xenografts growing in immunodeficient scid mice.
2. To determine conditions required for the selective accumulation of intravenously-administered biotinylated proteins and peptides to antibody-streptavidin pretargeted human tumor xenografts growing in immunodeficient scid mice.
3. To examine the host cellular infiltrate at tumor sites in mice following therapy with cytokines pretargeted to tumors by streptavidin-conjugated antibodies.
4. To examine the growth properties of tumors in mice treated with antibody-pretargeted cytokines.

Work Accomplished

We made significant progress in achieving objectives 1 and 2.

NR-LU-10 – Sterptavidin Immunoconjugate

This immunoconjugate was obtained from NEORx Corporation, and was shown to bind by flow cytometry to HT-29 cells that overexpress Ep-CAM antigen (not shown).

Biotinylation of Interleukin-2 (IL-2)

IL-2 was labeled through its carboxy-terminal cysteine according to manufacturer's instructions (Pierce). Excess biotin was removed by dialysis. The biotinylated IL-2 was purified and removed over an avidin column (Pierce) and eluted with 100 mM glycine, pH3.0. The final product was dialyzed against PBS with a final recovery of 25%, and was frozen into 200 µl aliquots at concentration of .228 mg/ml. A HABA assay was used to determine the molar biotin: IL-2 ratio.

Characterization of Biotinylated IL-2

IL-2 Receptor Binding. The binding of IL-2 species to the IL-2 dependent NK92 cell line known to express the high affinity IL-2 receptor was measured by flow cytometric analysis. The results are depicted in the table below.

IL-2 (nM)	MFI	Bt-IL-2 (nM)	MFI	% of native IL-2
1	21.6	1	16.5	76
5	22.6	5	16.4	73
20	23.3	20	16.8	72

These results indicate that the biotinylated species exhibits significant, but reduced binding to the IL-2 receptor.

T cell proliferation assays. Unmodified and biotinylated IL-2 were added at various concentrations to 200,000 human peripheral blood lymphocytes and incubated for 72 hours. ^{3}H thymidine was added to the cell cultures and incorporation was measured and extrapolated for proliferation. Half-maximal stimulation occurred at 0.005 – 0.01 nM IL-2, and at 0.1 – 0.5 nM biotinylated IL-2, respectively.

Cytotoxicity assays. In another set of experiments, human carcinoma cell line SK-OV-3 was labeled with ^{51}Cr and added to human lymphocytes that had been incubated in varying concentrations of unmodified or biotinylated IL-2. At 25: 1 effector: target ratios, 14% lysis of tumor was mediated by lymphocytes activated in 2000 IU unmodified IL-2, while equivalent levels of tumor lysis were seen using lymphocytes activated in 5000 IU biotinylated IL-2. Thus, biotinylation reduces the T-cell proliferative effects of IL-2, but has relatively little impact on the ability of this cytokine to activate lymphocytes for tumor lysis. When biotinylated IL-2 is admixed with NR-LU-10 – streptavidin, the resulting immunoconjugate retains an ability to activate lymphocytes to promote tumor lysis. In one experiment employing the HT-29 tumor cell line overexpressing Ep-CAM, the immunoconjugate promoted tumor lysis by human lymphocytes at 25:1 effector: target ratios (not shown). However, the immunoconjugate did not promote significant ADCC, presumably because the bulky streptavidin conjugation sites interfere with Fc domain interactions with lymphocyte Fc receptors.

In vivo experiments. Based upon the above studies we concluded that the biotinylated IL-2 possesed adequate properties for in vivo studies with NR-LU-10 – streptavidin.

KEY RESEARCH ACCOMPLISHMENTS IN YEAR 1

- Biotinylation of interleukin-2 (IL-2).
- Characterization of biotinylated IL-2 binding properties, T-cell activation properties and capacity to promote lymphocyte-mediated cytotoxicity of tumor cells.
- Demonstration that admixture of streptavidin-conjugated antibody with biotinylated IL-2 is associated with retention of antibody binding and IL-2 binding properties.

At the conclusion of year one of this support we felt the results warranted continuation of this line of research. The biotinylation of IL-2 led to a loss of IL-2 biological activity, but we felt this loss would be overcome by a decrement in host toxicity coupled with the concentration of the cytokine at tumor sites by antibody pretargeting.

In the second year of support we made continued progress in achieving objectives 1 and 2.

Biotinylated IL-2 exhibited significant, but reduced binding to the IL-2 receptor. In order to show that Bt-IL-2 bound to NRLU-10 – streptavidin on cells a second flow cytometric analysis was performed in which the antibody was incubated with HT-29 cells followed by the addition of the Bt-IL-2. The interaction was detected using an anti-human IL2-FITC-labeled antibody. Results from this experiment showed that the Bt-IL-2 could bind to the NRLU-10 – streptavidin bound to cells.

In vivo experiments. Based upon the above studies we concluded that the biotinylated IL-2 possessed adequate properties for in vivo studies with NR-LU-10 – streptavidin. In the first set of mouse experiments ^{125}I-labeled NRLU-10-streptavidin antibody was evaluated to determine its optimal tumor targeting timepoint. These studies showed that best tumor to organ ratios were achieved between the 24 and 48 h timepoints. This finding confirmed prior observations that 30 h is the optimal timepoint to inject Bt-IL-2. In the following experiments, NRLU10-streptavidin was again injected into cohorts of scid mice bearing HT-29 subcutaneous xenografts (250 mg) followed by the injection of a clearing agent 24 h later. This clearing agent was administered in order to remove any unbound NRLU10-streptavidin from the bloodstream. Thirty hours post-antibody injection, ^{125}I-labeled Bt-IL-2 was injected into the mice. Two additional cohorts(24 h and 48 h timepoints) were treated in the same manner, with the exception that the IL-2 that was administered was not biotinylated, this provided a negative control. Further controls included injecting both the IL-2 and Bt-IL-2 without antibody to compare its targeting alone. Other cohorts were treated with NR-LU-10 – streptavidin admixed ex vivo with equimolar concentrations of biotinylated IL-2 and sacrificed at the same time points. Tumors and normal organs were assayed for radioactive content and results used to calculate the % injected dose per gram of tumor or organ, and to calculate tumor: normal organ ratios. Results showed that under these pretargeting conditions the ^{125}I-Bt-IL-2 was successfully targeted to tumor sites as compared to pretargeted ^{125}I-IL-2 or either of these molecules administered alone (see attached figure). Although these results were encouraging tumor to normal organ ratios were not high.

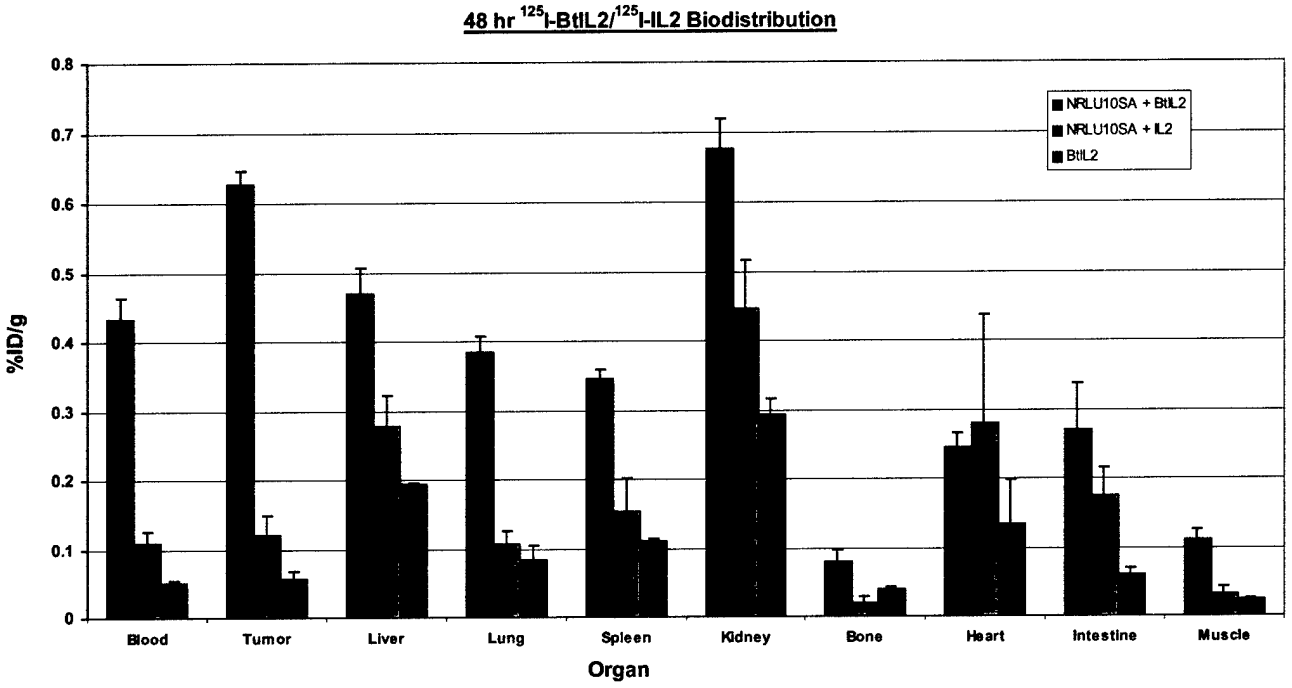


Figure 1 legend: ^{125}I-Biotinlyated IL-2 Biodistribution Study

In this experiment 400 μg of the NRLU-10SA antibody was first administered to HT29 tumor bearing SCID mice followed by injection with 100 μg of galactosylated-biotinyl-HSA, a clearing agent used to remove excess streptavidin-labeled antibody from circulation. The mice were then injected with 5 μg of either ^{125}I-BtIL2 or ^{125}I-IL2. One cohort received ^{125}I-BtIL2 alone as a second negative control. Shown is the average percent injected dose per gram of radiolabeled material in several organs and the blood.

KEY RESEARCH ACCOMPLISHMENTS YEAR 2

- Proof of concept that antibody pretargeting can promote the selective tumor retention of IL-2.

WORK ACCOMPLISHED IN YEAR 3

We had previously achieved objectives 1 and 2. Careful analysis of biodistribution results using antibody-strepavidin tumor targeting and biotin-IL-2 delivery to the pretargeted tumors (Objective 2) did not predict for therapeutic success due to innate deficiencies in the tumor targeting properties of the antibody-streptavidin conjugates. Accordingly, we devised an alternate strategy that utilizes a related pretargeting concept that addresses many of the deficiencies of the previously employed approach.

Initial in vivo studies showed that best tumor to organ ratios were achieved at 30 h following injection with NRLU1—streptavidin. NRLU10-streptavidin then was injected into cohorts of scid mice bearing HT-29 subcutaneous xenografts (250 mg) followed by the injection of a clearing agent 24 h later to remove any unbound NRLU10-streptavidin from the bloodstream. Thirty hours post-antibody injection, ^{125}I-labeled Bt-IL-2 was injected into the mice. Tumors and normal organs were assayed for radioactive content and results used to calculate % injected dose per gram of tumor or organ, and to calculate tumor: normal organ ratios. ^{125}I-Bt-IL-2 was successfully targeted to tumor sites as compared to pretargeted ^{125}I-IL-2 or either of these molecules administered alone, but tumor to normal organ ratios were not high enough to predict that the amount of Bt-IL-2 delivered to tumor site would be sufficient to induce tumor regression. However, these initial results demonstrated that the concept of antibody-pretargeted cytokine therapy continues to have merit and is worth pursuing if an improved pretargeting system can be identified, produced and implemented.

Our efforts then concentrated on the construction of bispecific antibody molecules in which two antibody domains bind the HER2/*neu* tumor antigen, and one or two additional domains bind a chelate (CHX-A") that can be conjugated to a cytokine. We successfully produced minibodies in various formats (see figure below). Anti-CHX-A" single-chain Fv molecules have been panned from a human phage display library; we plan to clone these scFv into the minibody constructs to create bispecific molecules with the desired tumor pretargeting characteristics. When this has been accomplished will conjugate IL-2 to CHX-A" and examine the potential value of this pretargeting approach.

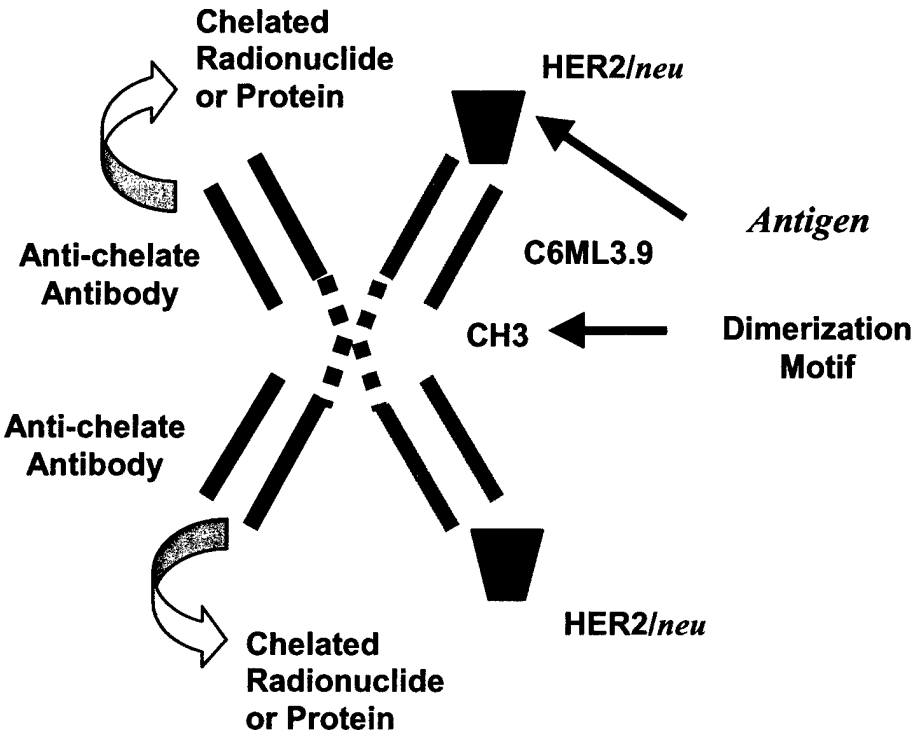


Figure 2. Design of New Pretargeting Vehicle

Thus far we have created an initial template containing antibody domains that we have extensively worked with in the past. One antibody contains anti-HER2/*neu* binding specificity and the other antibody has specificity for a different ligand, FcγRIII (which is the Fcγ receptor, CD16, with specificity for Fc domains of immunoglobulin G (Figure below).

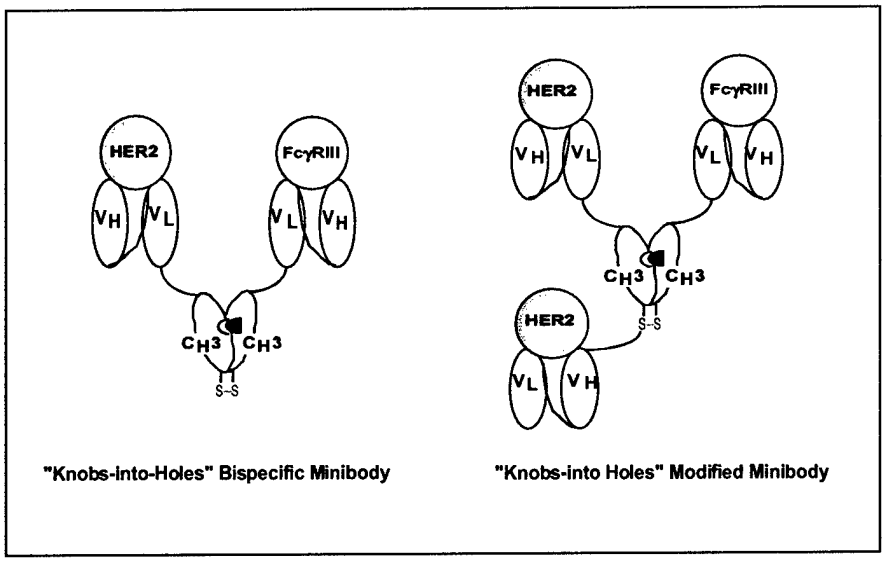
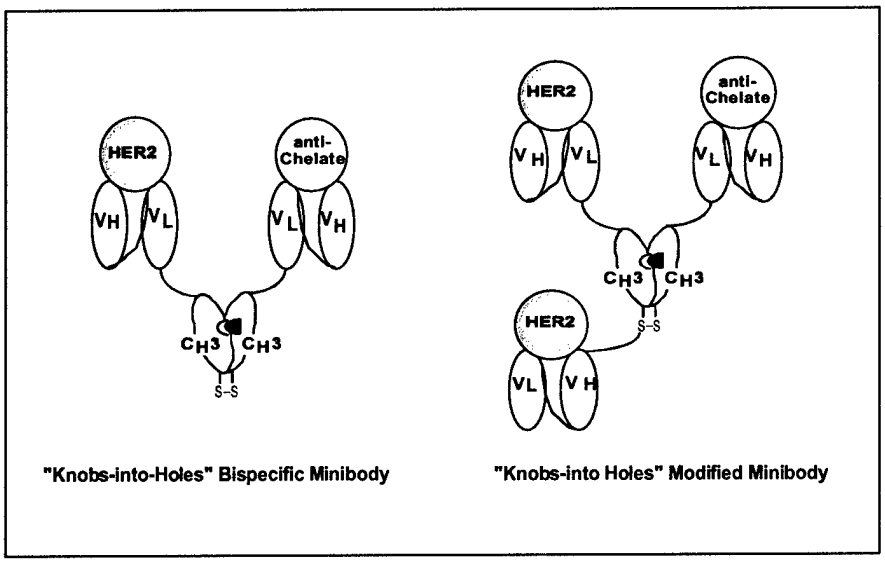


Figure 3. Schematic depicting the Structure of the two distinct minibody structures

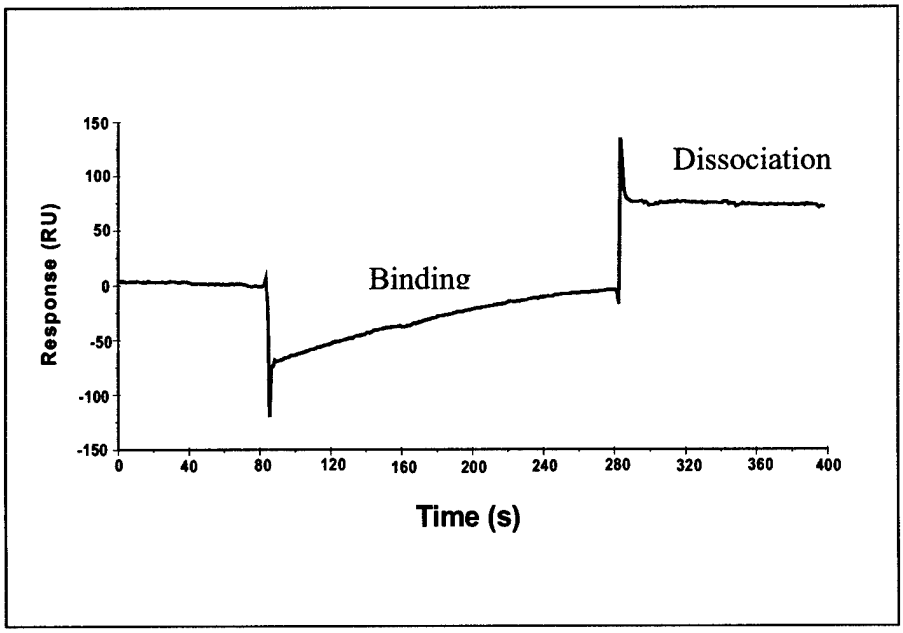
As is evident in this figure, both structures employ the "knobs-into-holes" concept, whereby heterodimerization based on affinities of CH3 domains is favored. Two distinct bispecific minibodies have been produced. The first is a dimeric molecule (left figure), while the other is trimeric, as depicted on the right side of the figure. Tetramers can be produced as well (not shown). Since the genes for these molecules are in hand, it is relatively straightforward to excise the anti-FcγRIII variable heavy and light chain genes and replace them with V_H and V_L genes encoding protein domains that bind to the metal chelate, CHX-A". To that end, we have obtained single-chain Fv molecules that bind to this chelate from a long-time collaborator, Dr. James Marks and intend to create bispecific minibodies that bind to HER2/*neu* and to CHX-A" once the anti-CHX-A" scFv have been affinity-matured (see Figure 4 below).

Figure 4. Bispecific Antibodies targeting HER2/*neu* and CHX-A"



We anticipate success in producing the bispecific minibodies schematized above. The anti-HER2/*neu* x anti-FcγRIII minibodies exhibit the desired binding properties in a plasmon sensor resonance assay (BIAcore, Pharmacia). Shown below (Figure 5) is a representative tracing employing the dimeric bispecific minibody (Figure 3, left).

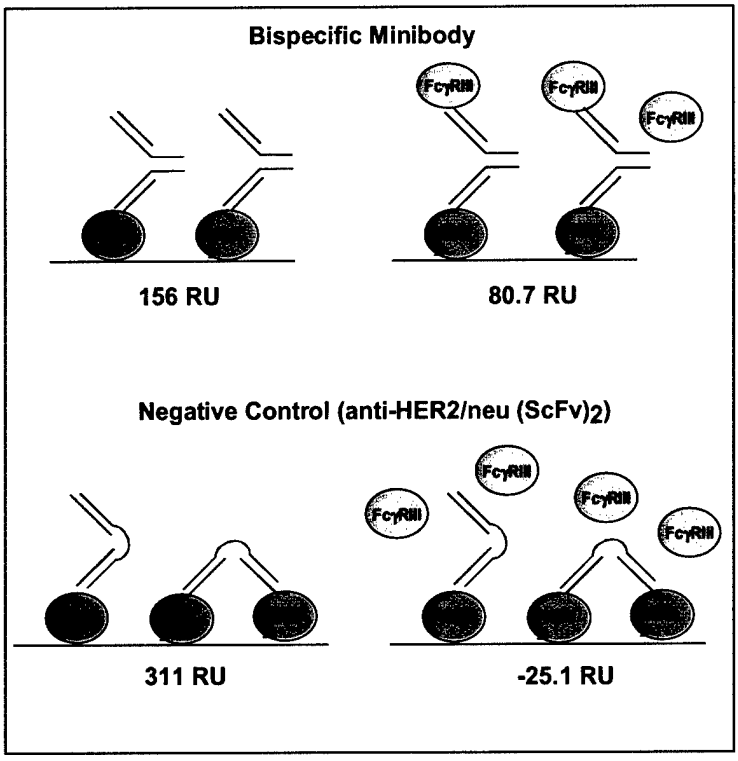
Figure 5. Anti-HER2/*neu* binding and slow dissociation of a dimeric, bispecific minibody



Similar results were demonstrated for binding to FcγRIII (not shown).

A novel "sandwich" based BIAcore assay was developed to investigate the capacity of the minibody to mediate bispecific binding. Figure 5 and Table 1 below show that the minibody, but not a monospecific antibody, was able to simultaneously bind to HER2/*neu* and to FcγRIII. Thus, these data support the idea that bispecific proteins can be produced and shown to mediate the desired binding properties. When the anti-CHX-A" variable heavy and light chain genes have been introduced into these structures, we intend to employ these molecules in tumors that overexpress HER2/*neu*; we have extensive experience with SK-OV-3 and with the breast cancer line MDA-MB-231-DYT2; both lines grow well in immunodeficient mice and express abundant HER2/*neu* on the cell surface.

Figure 6. Sandwich BIAcore assay demonstrates binding properties of a bispecific minibody



Sandwich assay demonstrates bispecific binding properties of anti-HER2/*neu* x anti-FcγRIII minibody

Structure	HER2 (RU)	CD16 (RU)	Bispecific Binding
YCMC	+ (75)	+ (98)	+
TriBi	+ (154)	+ (310)	+
KHYCMC	+ (635)	+ (162)	+
KHTriBi	+ (440)	+ (242)	+
Neg. Control	-	-	-

We intend to modify IL-2 by covalently adding a labeled metal species (probably Indium-111) to its carboxyl terminus using standard technology extensively employed in our laboratory. The ability of the labeled IL-2 to be selectively retained at antibody-pretargeted tumor sites then will be determined.

KEY RESEARCH ACCOMPLISHMENTS YEAR 4

- Development of new vehicles for antibody-directed pretargeted cytokine therapy.

CONCLUSION

We conclude that additional targeting vehicles and pretargeting strategies must be developed in order for the concept of antibody-pretargeted cytokine therapy to achieve its potential.

REPORTABLE OUTCOMES

L. Shahied, R.K. Alpaugh, G.P. Adams, H.H. Simmons, E.M. Horak, C.C. Shaller, D.B. Axworthy, A.R. Amoroso, and L.M. Weiner (2000) Pretargeting Mechanism for the Delviery of Interleukin-2 to Tumor Site. The 11th Annual International Conference on Antibody Engineering (Poster Presentation).

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LIST OF PERSONNEL

Louis M. Weiner, Principal Investigator
Calvin Shaller, Scientific Assistant

APPENDICES

None.